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Molecular epidemiology of the endemic multiresistance plasmid pSI54/04 of *Salmonella* Infantis in broiler and human population in Hungary

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ABSTRACT

Salmonella Infantis (SI) became endemic in Hungary where the PFGE cluster B, characterized by a large multiresistance (MDR) plasmid emerged among broilers leading to an increased occurrence in humans. We hypothesized that this plasmid (pSI54/04) assisted dissemination of SI. Indeed, Nal-Sul-Tet phenotypes carrying pSI54/04 occurred increasingly between 2011 and 2013 among SI isolates from broilers and humans. Characterization of pSI54/04 based on genome sequence data of the MDR strain SI54/04 indicated a size of ~277 kb and a high sequence similarity with the megaplasmid pESI of SI predominant in Israel. Molecular characterization of 78 representative broiler and human isolates detected the prototype plasmid pSI54/04 and its variants together with novel plasmid associations within the emerging cluster B. To test *in vitro* and *in vivo* pathogenicity of pSI54/04 we produced plasmidic transconjugant of the plasmid-free pre-emergent strain SI69/94. This parental strain and its transconjugant have been tested on chicken embryo fibroblasts (CEFs) and in orally infected day old chicks. The uptake of pSI54/04 did not increase the pathogenicity of the strain SI69/94 in these systems. Thus, dissemination of SI in poultry could be assisted by antimicrobial resistance rather than by virulence modules of the endemic plasmid pSI54/04 in Hungary.

1. Introduction

Salmonella Infantis has been the most prevalent serovar isolated from fresh poultry meat and broiler flocks all over in Europe (EFSA and ECDC, 2015) and in several other countries like Israel in 2007–2009 (Gal-Mor et al., 2010) and Japan 2000–2003 (Asai et al., 2006; Shahada et al., 2006). Concomitantly *S.* Infantis became the fourth most widespread serovar isolated from humans in the EU, behind *S.* Enteritidis, *S.* Typhimurium and monophasic *S.* Typhimurium 1,4,[5],12:i:- (EFSA and ECDC, 2015). Earlier *S.* Infantis has been frequently isolated from asymptomatic poultry (Asai et al., 2006; Shahada et al., 2006), and human patients in Japan (Murakami et al., 2007). It became the most dominant serovar in poultry and in the human population in Israel (Gal-Mor et al., 2010).

Dissemination of multidrug resistant (MDR) *S.* Infantis in Belgian poultry carrying a $bla_{\text{TEM-52}}$ plasmid has been reported by Cloeckaert et al. (2007). The first evidence for clonal spread of *S.* Infantis strains in broiler and in human populations with a large conjugative MDR plasmid (>168 kb) carrying a class 1 integron (containing the *aadA1* gene cassette for streptomycin/spectinomycin resistance) and the *tet*(A) gene for tetracycline resistance came from Hungary (Nógrády et al., 2007). Emerging strains characteristically showing nalidixic acid-streptomycin-sulphonamide-tetracycline (NalStrSulTet) resistance belong to the dominant PFGE cluster B (Nógrády et al., 2007, 2008). The representative strain SI54/04 of this cluster B has been sequenced and its large MDR plasmid, earlier estimated as >168 kb, has been determined as ~277 kb (Olasz et al., 2015). Our further studies have shown that these MDR *S.* Infantis strains are also endemic among broilers in

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several European countries, especially in Austria and Poland and belong to the same or closely related clones as the Hungarian cluster B (Nógrády et al., 2012). Furthermore, they also carry the same or very similar large MDR plasmid with class 1 integron and *tet*(A) gene.

Here we aimed at studying the recent epidemiology of MDR *S.* Infantis in Hungary by molecular characterization of the strains and of the emerging ~277 kb MDR plasmid designated as pSI54/04. Plasmid pSI54/04 of the emerging Hungarian broiler strain SI54/04 revealed high sequence similarity with the megaplasmid pESI of a human *S.* Infantis strain (Aviv et al., 2014) concerning its specific regions with functions in antimicrobial resistance, virulence, plasmid maintenance and transfer. Molecular typing of recent *S.* Infantis strains of broiler and human origin indicated that the PFGE cluster B carrying this MDR plasmid is still endemic and pSI54/04 might have contributed to the unprecedented spread of *S.* Infantis between 2000 and 2013 in the Hungarian broiler flocks and to a much lesser degree in humans.

2. Materials and methods

2.1. Strains of Salmonella Infantis and antimicrobial resistance phenotyping

A collection of 186 epidemiologically unrelated strains of *S.* Infantis was established, by choosing isolates with differing geographic origin, covering as much of Hungary as possible for the years of 2011–2013, containing approximately equal numbers of broiler and human isolates without causative links between them (Table S1). This basic collection was set up in order to represent the antimicrobial resistance profiles of *S.* Infantis in Hungarian broiler flocks and in humans. It provided a basis for comparative epidemiological and molecular studies for Hungary, keeping in mind that about 90% of poultry meat consumed is produced in this country. Broiler strains (n = 91) were provided by the Food and Feed Safety Directorate of the National Food Chain Safety Office (NEBIH), as part of the national *Salmonella* monitoring program, representing overwhelmingly the two major broiler breeds Ross 308 and Cobb 500. Human strains of *S.* Infantis (n = 95) were provided by the National Center for Epidemiology, representing sporadic clinical cases. Strains were stored at −80 °C in LB (Luria-Bertani) broth containing 10% glycerol.

Antimicrobial susceptibility testing of *S.* Infantis strains was performed by disc diffusion against the listed antimicrobial compounds: ampicillin (Amp), cefotaxime (Ctx), chloramphenicol (Chl), ciprofloxacin (Cip), kanamycin (Kan), nalidixic acid (Nal), sulfonamide compounds (Sul), tetracycline (Tet) and trimethoprim (Tmp). Results were interpreted according to Clinical and Laboratory Standard Institute (CLSI) guidelines and interpretive standards (CLSI, 2013). *S.* Infantis isolates with intermediate zone diameter values to respective antimicrobials were considered susceptible. *E. coli* ATTC 25922 was used as a reference strain.

2.2. Molecular characterization: pulsed-field gel electrophoresis, plasmid profiling and antimicrobial resistance genotyping

A reduced number of broiler (n = 31) and human (n = 47) strains of *S.* Infantis were selected for molecular analysis (Table S1). Selection of the strains was based on their antimicrobial resistance phenotype to represent the diversity of the existing resistance patterns. As a reference, two sequenced strains of broiler origin, SI69/94 and SI54/04 were also included (Olasz et al., 2015). They represented the pre-emergent plasmid-free pansensitive isolates from the 1990s and the emerging MDR strains from the 2000s carrying the plasmid pSI54/04 respectively (Nógrády et al., 2007, 2008; Olasz et al., 2015).

PFGE analysis was carried out according to the CDC PulseNet standardized *Salmonella* protocol using *Salmonella* Braenderup H9812 as a molecular standard. PFGE-generated DNA profiles were entered into the Fingerprinting II Software (Bio-Rad Laboratories, Ventura, CA, USA). Cluster analysis was performed by the unweighted pair-group method (UPGMA) with arithmetic means. DNA sequence relatedness was calculated on the basis of the Dice's coefficient. A 1.0% position tolerance and 1.5% optimization setting were applied.

Plasmid preparation was carried out using the alkaline lysis method of Kado and Liu (1981), and plasmids were separated in 0.75% agarose gel in a vertical system. The approximate sizes of plasmids were estimated by comparing them with the reference plasmids of *E. coli* V517 (2.0–53.7 kb) and *E. coli* MD112 (168 kb) using the Quantity One software (Bio-Rad Laboratories).

Resistance gene patterns of the above selected strains were identified by AMR05 PCR-microarray (http://alere-technologies.com/en/products/lab-solutions/amr-ve-genotyping.html), designed to detect among others several plasmidic genes conferring resistance to aminoglycosides, β-lactams (including extended spectrum β-lactams), quinolones and tetracyclines, as well as genes associated to class 1 and class 2 integrons (Batchelor et al., 2008). Array spots were read with ArrayTube Reader ATR03 and the signals were detected and analyzed using IconoClust 2 software, with the positive threshold values set at ≥ 0.4.

2.3. Sequence analysis of specific regions and PCR-typing of plasmid pSI54/04

The deposited genome contigs of the emerging strain SI54/04 (Olasz et al., 2015) were used for the extraction of specific regions of plasmid pSI54/04 with functions in antimicrobial resistance, virulence, plasmid maintenance and transfer. As a reference for the assembly of these plasmidic regions, the corresponding contigs (ASRF01000099 - ASRF01000108) of the published megaplasmid pESI from an Israeli MDR strain of human *S.* Infantis were used (Aviv et al., 2014). Bioinformatic analysis was performed by Geneious 9.0.5 software package (Biomatters Ltd), for pairwise alignment the inbuilt application Geneious Alignment was used under default settings.

In order to identify pSI54/04 and its possible variants, we developed a PCR typing system with primers designed to identify marker genes from each specific regions of the prototype plasmid pSI54/04. Primers and the corresponding target genes are presented in Table 1. Primers were tested for specificity against the NCBI nucleotide database by using BLAST. Broiler and human strains of *S.* Infantis that carried a large plasmid of >168 kb were subjected to pSI54/04 typing by using the above PCR system. The emerging strain SI54/04 and the plasmid-free pre-emergent strain SI69/94 were included as positive and negative controls. Simplex PCR reactions were performed in a final volume of 25 μl, containing 0.6 u of PCRBIO Taq DNA polymerase, 5 μl of 5× PCRBIO Reaction buffer, 400 nM of each primer (Sigma-Aldrich), and 2 μl of template DNA. Amplifications were performed in a BIO-RAD iCycler PCR system, and reaction conditions were uniform for all set of primers: 94 °C for 3 min, 35 × 94 °C for 30 s, 60 °C for 1 min, 72 °C for 30 s and a final extension at 72 °C for 10 min. Detection of genes *tet*(A), *intI1* and *sul1* considered as prime markers for pSI54/04, were performed by the above PCR-microarray (Batchelor et al., 2008). Plasmid incompatibility of pSI54/04 was determined by PCR-based replicon typing (PBRT) developed by Carattoli et al. (2005).

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